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13. ABSTRACT *(Maximum 200 Words)*

Our research is based on our observation that the β_{II} isotype of tubulin is found in the nuclei of breast cancer cells. Our goals are to learn the function of β_{II} and to design a novel anti-tumor drug that will target β_{II}. We have found that β_{II} enters the nucleus without going through the nuclear membrane. Instead, β_{II} binds to a nuclear component after the nucleus has disintegrated during mitosis and then remains in the nucleus when the nucleus re-forms after mitosis. The process by which β_{II} is localized to the nucleus is specific for β_{II}; neither β_{III} nor β_{IV} do this. It is possible that the nuclear component to which β_{II} binds could be a novel target for anti-tumor drugs. We have found that vinblastine and taxol, two well-known anti-tumor drugs, cause β_{II} to be expelled from the nucleus. This raises the possibility that this may be part of their mechanism of action. We have also synthesized a derivative of colchicine bound to a peptide with a nuclear localization sequence; this peptide is likely to target nuclear tubulin.

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FOREWORD

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Richard F. Ludueña   May 29, 2000
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PI - Signature   Date

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INTRODUCTION

The research reported here is based on the observation that one of the isotypes of tubulin, β_{II}, is found in the nuclei of a wide variety of cancer cells, including breast, prostate, and lung. We have found nuclear β_{II} not only in cultured cancer cells but also in breast cancer *in situ*. We have also found β_{II} in the nuclei of cultured rat kidney mesangial cells. In contrast, we do not see nuclear β_{II} in normal cells. We have two overall aims in this research: 1) to identify the function of nuclear β_{II}; and 2) to design a drug that will target nuclear β_{II}. Our overall hypothesis in the first aim is that nuclear β_{II} somehow aids in rapid cell proliferation; our objective is to find out how. We have microinjected monoclonal antibodies specific for individual tubulin isotypes into both breast cancer and rat kidney mesangial cells and we have found that they have no apparent effect on cell viability and function. Microinjection of antibodies into mesangial cells gave similar results. Accordingly, we are developing a variety of approaches to test this hypothesis. We have found that the localization of β_{II} in nuclei does not require that it enter the intact nucleus; rather, nuclear β_{II} binds to a nuclear component during mitosis when the nucleus has disintegrated. As the nucleus re-forms it incorporates β_{II} into it. The process, whatever it is, is specific for β_{II}; neither β_{III} nor β_{IV} will enter the nucleus. The hypothesis underlying the second aim is that an anti-tubulin drug that has been modified by addition of a moiety that would be directed toward the nucleus may be particularly toxic to cancerous cells that contain nuclear tubulin. Accordingly, we have attempted to link the anti-tubulin drug colchicine to such a moiety, in one case to estradiol, in the other to a peptide containing a nuclear localization sequence.

BODY

TASK 1: MICROINJECTION OF ISOTYPE-SPECIFIC ANTIBODIES INTO BREAST CANCER AND NORMAL CELLS

Rationale:

The research described here is based on the finding that the β_{II} isotype of tubulin occurs in the nuclei of a variety of cancer cells, including breast cancer cells. The specific goal for Task 1 is to determine the function of nuclear β_{II}. The idea is to microinject antibodies specific for each tubulin isotype into cells and see what cellular functions are disrupted. As will be described below, microinjection did not give unequivocal results, hence, we are developing new approaches to address this question.

Experimental Results:

Microinjection of the Isotype-Specific Antibodies

We microinjected breast cancer cells with monoclonal antibodies specific for the β_I, β_{II}, β_{III} and β_{IV} isotypes. We saw no change in their viability. In order to carry out this experiment in a system more focused on nuclear β_{II}, we turned to rat kidney mesangial cells. The reason is that the mesangial cells contain β_{II} almost entirely in their nuclei, whereas breast cancer cells contain β_{II} in both the nuclei and the cytoplasm (1). Our rationale was that if microinjection of anti-β_{II} into mesangial cells had an effect, then that effect was likely to be attributable to interfering with nuclear β_{II} as opposed to cytoplasmic β_{II}. We microinjected them during interphase and mitosis. Microinjection was done into the cytoplasm and the nuclei. We found that microinjection of anti-β_{II} had no effect at all on the mesangial cells, including not only their viability and ability to form mitotic spindles but also their ability to undergo cAMP-induced shape change, a process that can be blocked by microtubule-inhibitory drugs. Microinjection of antibodies against β_{III} and β_{IV} had no effect either. The only effect was seen with micro-injection of anti-β_I, which caused the cells to undergo unusual shape changes when cAMP was added. This is likely to reflect the fact that β_I is the most abundant isotype in the cytoplasm of mesangial cells rather than arising from a specific function of β_I.

The fact that microinjection of isotype-specific antibodies into cells did not have any major effect does not mean that specific isotypes do not have particular functions, nor that nuclear β_{II} has no significance. It is possible that the microinjected antibodies were not sufficiently concentrated to interact with their isotypes. There could also be a question of timing, since the antibodies can only last for a certain length of time before the cell degrades them. There may be specific times during the cell cycle during which the isotype differences become important; perhaps the effective concentration of the antibody at those times in the vicinity of its antigen is not sufficiently high to interact

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with the isotypes. At any rate, we decided to address the question of the functional significance of tubulin isotypes and, specifically, of nuclear β_{II}-tubulin, using other approaches. We are presently trying to understand what it is about the β_{II} isotype that causes it to enter the nuclei. Solving this problem may explain the function of nuclear β_{II}.

Confocal Microscopy of Breast Cancer and Normal Breast Epithelial Cells

Our results, using normal immunofluorescence microscopy, have shown that β_{II} occurs in the nuclei of breast cancer cells and in lesser amounts in the nuclei of MCF-10F cells, that are supposedly non-transformed breast cancer cells (Figure 1). Even in our best micrographs, however, it was not clear that, in breast cancer cells, the other isotypes were absent from the nucleus. It took us a long time to locate a viable system for confocal microscopy, but we now have an arrangement with Dr. Victoria Froehlich, Assistant Professor in the Department of Cellular and Structural Biology at the Health Science Center who supervises the Optical Imaging Core Facility. This facility has an Olympus FV500 Confocal Scanning System equipped with argon and red and green helium-neon lasers, based on an IX70 inverted microscope. Very recent results using this microscope has shown clearly that the β_I, β_{III} and β_{IV} isotypes, all of which are expressed by MCF-7 breast cancer cells, are absent from their nuclei (Figures 2-4). They are also absent from the nuclei of MCF-10F cells. Thus, it appears very clear that the nuclear localization of β_{II} is specific for that isotype.

Microinjection of Fluorescently Labeled Tubulin Isotypes into Mesangial Cells

Since mesangial cells have only low levels of cytoplasmic β_{II}, we have begun this approach using these cells. As previously reported, we microinjected these cells with fluorescently labeled $\alpha\beta_{II}$ tubulin. $\alpha\beta_{II}$ is isotypically purified tubulin, that is, a tubulin dimer whose β subunit is β_{II}, as opposed to isotypically unfractionated tubulin, which, in our source of bovine brain, is a mixture of $\alpha\beta_I$, $\alpha\beta_{II}$, $\alpha\beta_{III}$ and $\alpha\beta_{IV}$ dimers (2). As previously reported, the fluorescently labeled $\alpha\beta_{II}$ localized to the nucleus, and in the same pattern as we found for the cell's own $\alpha\beta_{II}$, namely, concentrated in the nucleoli (1). We found, however, that a mitotic cycle had to pass before the $\alpha\beta_{II}$ entered the nuclei. $\alpha\beta_{II}$ that was microinjected during mitosis also entered the nucleus. These results imply that the process by which $\alpha\beta_{II}$ does not involve entering the intact nucleus, but rather that it must bind to a nuclear component during mitosis, when the nucleus has disintegrated, and that $\alpha\beta_{II}$ is present in the re-forming nucleus.

We then went on to microinject the cells with fluorescently labeled $\alpha\beta_{III}$ and $\alpha\beta_{IV}$. These did not enter the nucleus, no matter when they were injected. Thus, this implies that the mechanism of nuclear entry is specific for $\alpha\beta_{II}$. Presumably, therefore, there is a nuclear component that binds only to β_{II} and not to the other isotypes.

Post-translational Modification of Nuclear β_{II}-Tubulin

One possible explanation for how β_{II} localizes to the nucleus is that it undergoes a different type of post-translational modification than does non-nuclear β_{II}. This is not a trivial possibility since the tubulin molecule undergoes more post-translational modifications than most other proteins; these include detyrosinolation and retyrosinolation of α, removal of the last two residues from α, acetylation of α, phosphorylation, palmitoylation, and polyglutamylation of both α and β, and polyglycylation of β (3). We have previously shown that the α in nuclear $\alpha\beta_{II}$ is tyrosinolated (1). We have recently subjected a nuclear extract to gel electrophoresis followed by immunoblotting using antibodies against polyglutamylated and phosphorylated proteins. We found very clear evidence that nuclear β_{II} is not polyglutamylated; our results also suggested that nuclear β_{II} may be phosphorylated at a tyrosine residue. This latter result was not unequivocal, however, since we could not be sure that the phosphorylated protein was β_{II}. We will be continuing these studies.

Identification of Nuclear Proteins Binding to β_{II}

This has so far been disappointing. We have used co-localization and immunoprecipitation to attempt to identify proteins that bind to β_{II}. We previously reported that vault ribonucleoprotein co-localized with β_{II} in the nuclei of mesangial cells. We have now found that our anti-vault antibody may also bind to tubulin, casting the results of the co-localization experiments into doubt. We also looked for co-localization of nuclear β_{II} with tau, microtubule-associated protein 4 (MAP4), and adenomatous polyposis coli protein (APC). None of these proteins co-localized with nuclear β_{II}.

We also attempted to approach this question by immunoprecipitation of a nuclear extract with anti-β_{II}. We found that 4 bands were obtained; however, the same bands were obtained by immunoprecipitation with anti-β_I and anti-β_{IV}, so there was no specificity with β_{II}. We now intend to do immunoaffinity chromatography with an $\alpha\beta_{II}$ column.

Double-label Immunofluorescence of the Mitotic Spindle

We have been exploring the hypothesis that the tubulin isotypes play different roles during mitosis and that nuclear localization of β_{II} allows it to play a unique role during mitosis. We have occasionally observed that β_I, β_{II} and β_{IV} do not co-localize exactly in the spindle and midbody. We are planning some confocal microscopy experiments to examine this at higher resolution. One specific hypothesis that we wish to test is that the different isotypes distribute differently among the microtubules of the mitotic spindle. If one isotype is clustered around the kinetochore, for example, it could help attract microtubules from the centrosome to the kinetochore; this would make the process of mitosis more rapid and accurate and would allow for faster cell proliferation. We intend to test this hypothesis.

In collaboration with Dr. Mohan Natarajan of the Radiology Department, we are looking at the effect of ionizing radiation on MCF-7 breast cancer cells. MCF-7 cells were subjected to a burst of ionizing radiation and then allowed to incubate for 24 hours. Cell proliferation was not markedly affected, compared to control cells that had not been irradiated (Table 1). The colchicine binding activity of the cell extract at 3 hours increased by 13%, however, at 24 hours, the irradiated cells had 44% less colchicine binding activity than did the control. This suggests that the MCF-7 cells have switched isotypes in response to the irradiation, perhaps switching to one that binds less colchicine, such as $\alpha\beta_{III}$. We will be examining nuclear β_{II} localization and also isotype composition of the irradiated breast cancer cells. One hypothesis about nuclear β_{II} is that the nucleus acts as a storehouse for tubulin to protect it from free radicals that are likely to be generated by ionizing radiation.

Effect of Drugs on Nuclear β_{II}

We have found that nuclear β_{II} is very sensitive to drugs; the effect of drugs, however, is surprising. We have found that vinblastine causes disappearance of cellular microtubules and also of nuclear β_{II}. Taxol causes disappearance of nuclear β_{II} but also induces bundling of microtubules in the cytoplasm. We do not yet know how to interpret these findings, but they have two implications. First, since β_{II} disappears from the nucleus without the cell progressing through mitosis, there must be a way for β_{II} to exit the intact nucleus even if it cannot enter it. Second, this finding could relate to either the role of nuclear β_{II} or its ability to serve as a target for anti-tubulin drugs.

TASK 2: SYNTHESIS AND CHARACTERIZATION OF A STEROID-COLCHICINE DERIVATIVE THAT TARGETS THE NUCLEUS AND BINDS TO TUBULIN

This task was scheduled to be performed in the first year of the grant, but the reviewers were very skeptical about its feasibility, so we postponed, however, in view of the previous finding that the distribution of β_{II} within the nucleus was correlated with resistance to estrogen, we decided to go ahead and attempt to make this compound (Scheme 1). The procedure was as follows: a slurry of β-estradiol (12.6 mg, 0.05 mmol), triphosgene (14.8 mg, 0.05 mmol) and potassium carbonate (4 mg, 0.03 mmol) in dichloromethane (4 mL) was stirred at room temperature under nitrogen for 2 hours. A solution of deacetylcolchicine (18 mg, 0.05 mmol) in dichloromethane (2 mL) was added and stirring of the resulting yellow slurry was continued overnight. The slurry was partitioned between dichloromethane and water, the organic layer separated, washed with water, dried over sodium sulfate, filtered and evaporated to give a brown residue. Proton NMR indicated formation of the NHC(O) bond but further analysis using high resolution mass spectrometry gave a molecular weight of 643.3027. The calculated molecular

weight should have been 654.3069. The 11 mass units difference indicates that there was problem with the synthesis.

TASK 3: SYNTHESIS AND CHARACTERIZATION OF A PEPTIDYL-COLCHICINE DERIVATIVE THAT TARGETS THE NUCLEUS AND BINDS TO TUBULIN

Background:

We synthesized the following peptide:

KRPRPCGMNK*EARKTKK

(* indicates that this lysine residue is labeled with fluorescein).

We showed previously that the peptide, when microinjected into rat kidney mesangial cells, began to accumulate in the nucleus within two hours after microinjection. After 20 hours, most of the peptide was in the nucleus.

We intended to make the peptidyl-colchicine derivative by reacting the peptide with deacetylcolchicine, but deacetylcolchicine is no longer commercially available. We therefore had to devise a synthesis for the peptide, beginning with colchicine. The synthesis (Scheme 2) was as follows:

Synthesis of Trimethylcolchicinic Acid:

A solution of colchicine (3.01 g, 8 mmol) in 4:3 concentrated HCl and methanol (70 mL) was refluxed for 48 hours. The solution was allowed to cool to room temperature after which a rinsing of the reaction flask was added. Neutralization of the pH with solid sodium carbonate gave a yellow flocculent precipitate which was extracted with dichloromethane. The aqueous layer was extracted and the combined organics dried by passage through sodium sulfate and evaporated to give crude trimethylcolchicinic acid. The structure was confirmed by ^{1}H NMR.

Synthesis and Purification of Deacetylcolchicine:

The next step was to methylate the trimethylcolchicinic acid with diazomethane. For this, trimethylcolchicinic acid (2.277 g) in dichloromethane (50 mL) cooled to 0-5 °C was mixed with a solution of KOH (16 g, 0.29 mol)) in 16:13 ethanol/water, and a solution of diazald™ (16g, 0.075 mol) in ether (144 mL). The reaction was distilled until the distillate was colorless. The reaction was allowed to come to room temperature and evaporated to give a brown residue that was dissolved in dichloromethane and evaporated again to remove excess diazomethane. The product was identified by ^{1}H NMR. This method gave a 1:2.5 mixture of deacetylcolchicine and isodeacetylcolchicine. Several

procedures were used in order to purify the deacetylcolchicine. All of them involved chromatography on silica gel (4,5).

Synthesis of *N*-4-maleimidobutyroyldeacetylcolchicine:

Once the deacetylcolchicine was purified it was derivatized to generate *N*-4-maleimidobutyroyldeacetylcolchicine. In one method, deacetylcolchicine was reacted with γ-maleimidobutyric acid in oxalyl chloride. The second method involved reacting deacetylcolchicine with γ-maleimidobutyric acid N-hydroxysuccinimide ester. The first two methods gave the purest product. The other methods involved using 1-hydroxybenzotriazole as a catalyst with or without dicyclohexylcarbodiimide. These generated the desired product, but with some by-products as well.

Synthesis of the Peptidyl-deacetylcolchicine Derivative:

The next step was to couple *N*-4-maleimidobutyroyldeacetylcolchicine to the above-mentioned peptide. For this, acetic acid (10%, 50 μL) was added to a solution of the peptide (0.5 mg, 0.2 μmol) in water (0.4 mL) and the resulting solution stirred for five minutes at room temperature. A solution of *N*-4-maleimidobutyroyldeacetylcolchicine (0.11 mg, 0.2 μmol) in acetonitrile (150 μL) was then added. Samples were withdrawn at various times and analyzed by HPLC. Analysis by MALDI indicated that the products were not pure. The largest product was identified as:

PRPC#GMNK*EARKTKK,

where # corresponds to the deacetylcolchicine attached to the cysteine residue and * is the fluorescein. It is clear that the peptide underwent partial hydrolysis during the coupling reaction, losing the first two residues. However, the truncated peptide still retains a nuclear localization sequence and should be able to localize to the nucleus. We intend to microinject it into cells shortly.

KEY RESEARCH ACCOMPLISHMENTS

- The process by which β_{II}-tubulin enters the nucleus of cells does not involve transportation into the nucleus but rather that it binds to a nuclear component during mitosis after nuclear disintegration; β_{II} then remains in the nucleus after the nucleus reforms.

- The process by which β_{II} enters the nucleus is specific for β_{II}. Neither β_{III} nor β_{IV} enter the nucleus.

- Nuclear β_{II}-tubulin is not polyglutamylated; in that respect it differs greatly from brain tubulin.

- Ionizing radiation causes a large change in tubulin properties in breast cancer cells. This could reflect a change in tubulin isotype composition.

- Vinblastine and taxol induce disappearance of nuclear β_{II}-tubulin.

- We have synthesized a derivative of colchicine attached to a peptide containing a nuclear localization sequence.

REPORTABLE OUTCOMES

1. Manuscripts, abstracts, presentations:

The research supported by this grant has led to two abstracts:

Walss, C., Barbier, P., Banerjee, M., Bissery, M.C., Lanieña, R.F. & Fellous, A. (2000) Nuclear tubulin as a possible marker for breast cancer cells. *Proc. Amer. Assoc. Cancer Res.* **41**, 553.

Ludueña, R.F., Banerjee, M., Moore, M., Walss, C., Barbier, P., Bissery, M.C. & Fellous, A. (2000) Nuclear β_{II}-tubulin as a marker and therapeutic target in breast cancer. Era of Hope Meeting.

This research has also led to two presentations:

The first abstract was presented at the annual meeting of the American Association for Cancer Research in San Francisco, CA, on April 4, 2000. Although originally intended to be presented as a poster, we were invited to present it as a platform talk in a minisymposium session.

The second abstract will be presented at the Era of Hope Meeting in Atlanta, GA, on June 11, 2000.

2. Funding Applied for Based on Work Supported by this Award

We submitted a grant to the National Institutes of Health Entitled "Nuclear β_{II}-Tubulin and the β_{IV}-Actin Link". Although the research proposed in this grant did not involve breast cancer cells, the research was based in part on results obtained under the auspices of this grant. The grant was not funded. I intend to revise the grant and re-submit it.

CONCLUSIONS

The basic finding on which this grant is based is that many cancer cells, including breast cancer cells contain the β_{II} isotype of tubulin in their nuclei. This year we have learned that the entry of β_{II} into nuclei only can happen during mitosis and that the process is specific for β_{II}. We have also observed that anti-tumor drugs can affect nuclear β_{II}. We have synthesized a compound that may target nuclear β_{II}. In short, the research results obtained this year have the following implications. First, they could lead to discovery of new drug targets, namely, the components of the mechanism by which β_{II} enters the nuclei. Second, they could lead to a deeper understanding of the mechanism of action of anti-tumor drugs. Third, we have synthesized a compound that may specifically target nuclear β_{II}. If this compound fulfils the purpose behind its design, it may lead to the development of some novel anti-tumor drugs.

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APPENDIX

Table 1. Effect of Ionizing Radiation on Colchicine-Binding Activity of MCF-7 Breast Cancer Cell Extracts

Ionizing Radiation (units)	After Three Hours in Culture		After 24 Hours in Culture	
	Total Prot (μg)	DPM	Total Prot (μg)	DPM
0	504	4650 ± 416	802	6600 ± 132
1	459	5400 ± 44	882	6801 ± 252
10	377	5264 ± 794	725	3723 ± 118

Batches of MCF-7 breast cancer cells were treated subjected to either 0, 1 or 10 units of ionizing radiation. They were then allowed to grow in culture for either 3 or 24 hours. At the indicated times, cells were harvested and homogenized in the presence of a cocktail of protease inhibitors (benzamidine (5 mM), TAME (2 mM), SBT1 (0.1 mg/mL), aprotinin (0.2 μg./mL), leupeptin (1 μg/mL), antipain (1 μg/mL), and PMSF (1% (v/v))). In each cell homogenate the total protein was determined. An aliquot of each homogenate was diluted to 0.75 mg protein/mL and was incubated with 50 μM [^{3}H]colchicine for 1 hour at 37 °C. Reactions were filtered and the radioactivity of the filters determined (6).
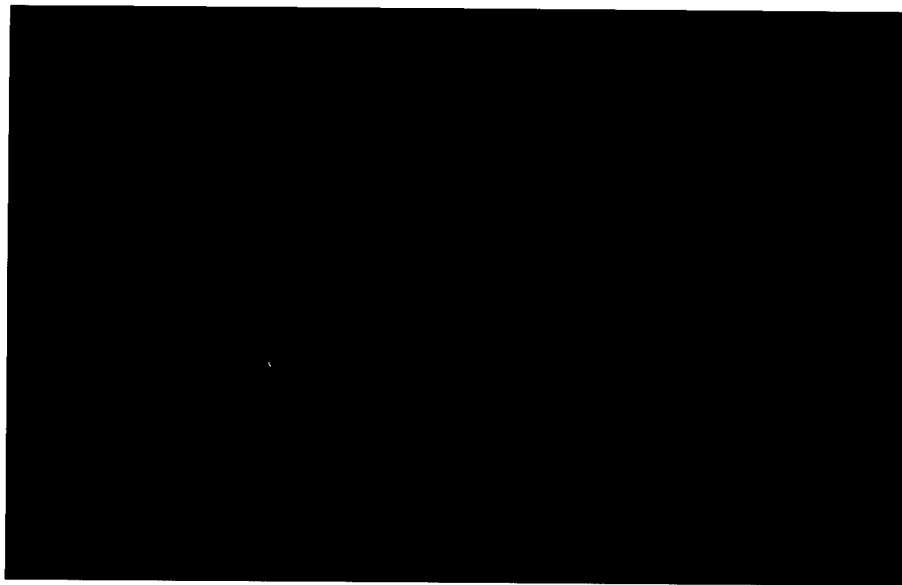
Figure 1. Comparison of transformed and non-transformed breast cancer cells.
Non-transformed breast epithelial MCF-10F cells (*left*) and Calc18 breast cancer cells
(*right*) were treated with an antibody to β_{II}-tubulin. Indirect immunofluorescence
microscopy with rhodamine was used to examine the cells (1). Notice the absence of
nuclear β_{II} in the MCF-10F cells and its presence in the cancerous cells.

Figure 2. MCF-7 Cells Treated with Antibody to β_I-Tubulin. Notice the absence of
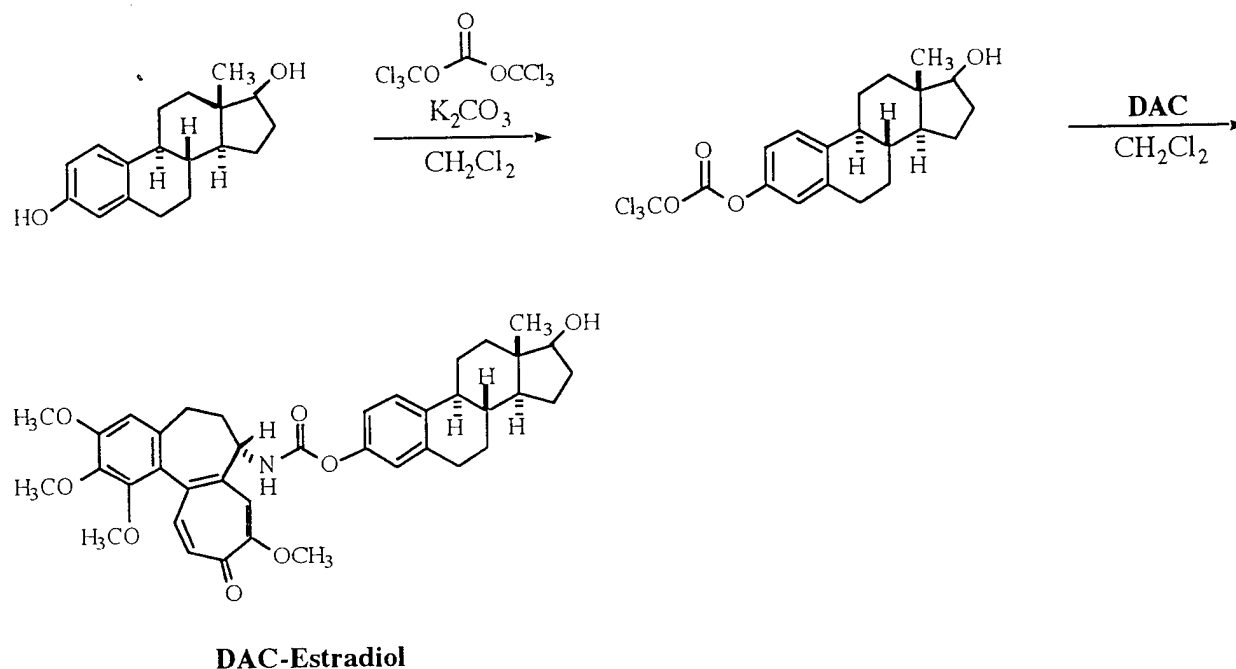β_I in the nuclei.

Figure 3. MCF-7 Cells Treated with Antibody to β_{III}-Tubulin. Notice the absence of β_{III} in the nuclei.


Figure 4. MCF-7 Cells Treated with Antibody to β_{IV}-Tubulin. Notice the absence of β_{IV} in the nuclei. In this figure indirect immunofluorescence was done with fluorescein.

Scheme 1

DAC-Estradiol

Scheme 1. Synthesis of Steroid-Colchicine Derivative. The scheme shows the mechanism by which deacetylcolchicine (**DAC**) can be added to estradiol to generate the derivative **DAC-Estradiol**.

Scheme 2
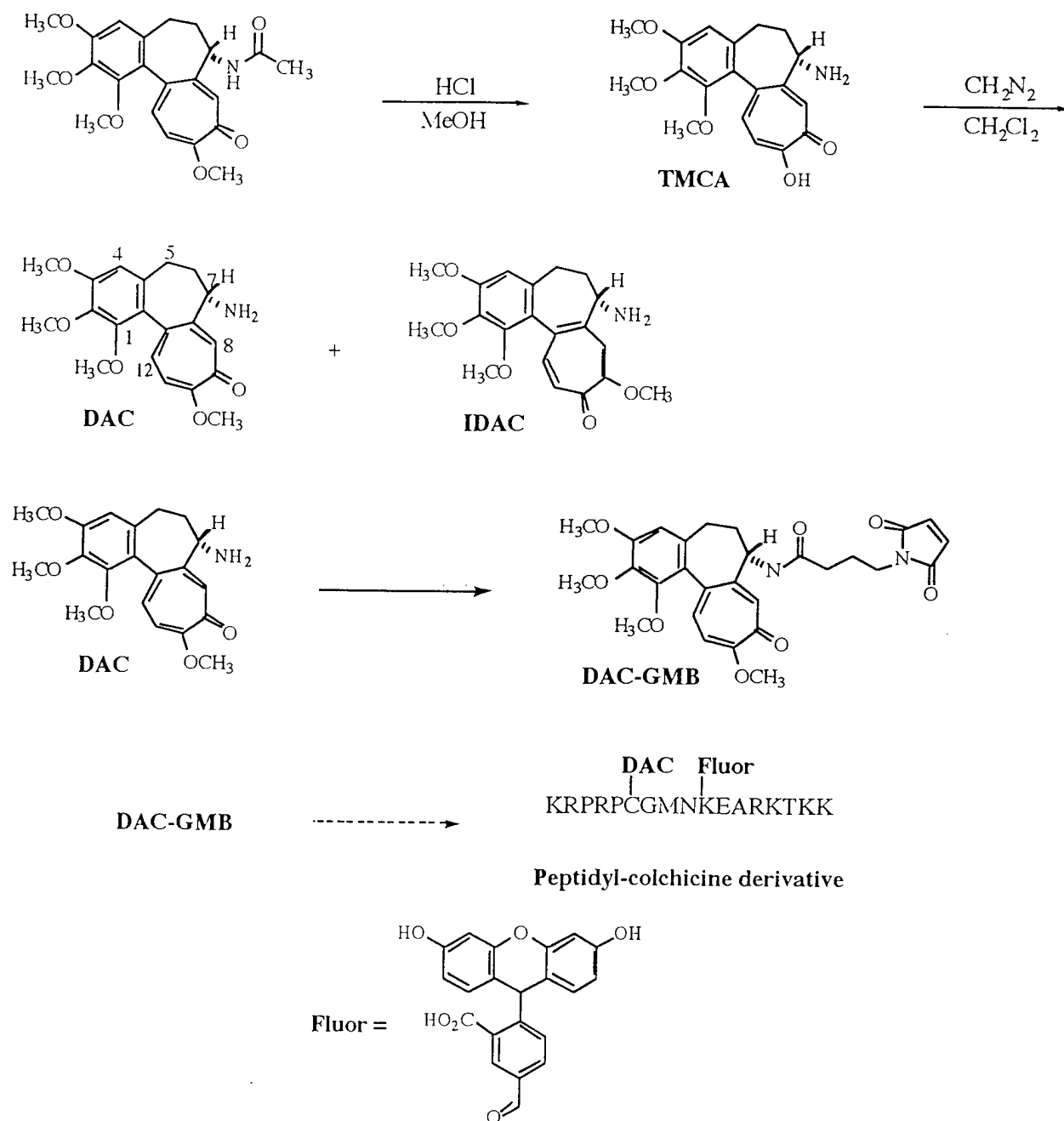
Scheme 2. Synthesis of Peptidyl-Colchicine Derivative. The scheme shows how colchicine is converted into trimethylcolchicinic acid (**TMCA**), which is in turn methylated to give a mixture of deacetylcolchicine (**DAC**) and isodeacetylcolchicine (**IDAC**). The **DAC** is separated from **the IDAC** and then converted into *N*-4-maleimidobutyroyldeacetylcolchicine (**DAC-GMB**). The **DAC-GMB** is then reacted with the peptide to generate the peptidyl-colchicine derivative.